JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Continuous Hot Pressurized Solvent Extraction of 1,1-Diphenyl-2-picrylhydrazyl Free Radical Scavenging Compounds from Taiwan Yams (*Dioscorea alata*)

PO-YEN CHEN,† YU-XUN TU,† CHENG-TAR WU,† TING-TING JONG,§ AND CHIEH-MING J. CHANG*,†

Departments of Chemical Engineering and Chemistry, National Chung Hsing University, 250 Kuokuang Road, Taichung, Taiwan, Republic of China

This study investigates a semicontinuous hot pressurized fluid extraction process and the scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical of the extract from Taiwan yams (*Dioscorea alata*). Liquid−liquid extractions were preliminarily employed to generate six fractions, initially extracted by ethanol. Then, the aqueous solution of dried crude ethanol extract was sequentially fractionated by hexane, chloroform, ethyl acetate, and *n*-butanol. The EC_{50} value was defined as the UV absorption of DPPH concentrations sufficiently decreased to 50% of the original value. It was found that all peel portions have a better effect on scavenging of the DPPH free radical than meat portions, especially for the ethyl acetate partition of the peel portion of Tainung #2 yam. Its EC_{50} value (14.5 μg mL^{-1}) was even lower than that of ascorbic acid (21.4 μg mL^{-1}). Furthermore, semicontinuous hot pressurized ethanol was superior to hot pressurized water in extracting the compound scavenging the DPPH radical from the *Purpurea-Roxb* peel. The recovery of four unknown compounds corresponded to the scavenging ratio of DPPH free radical in the hot pressurized ethanol extract. Finally, three-level and four-factor experimental design revealed that ethanol ratio and temperature were the most effective factors in order. Conditions of 80% of aqueous ethanol, 20.0 kg/kg solid ratio, 180 psig (1.342 MPa), and 100 °C were preferred to extract those antioxidants from the yam peel.

KEYWORDS: ***Dioscorea alata*; DPPH free radical; hot pressurized water; experimental design**

INTRODUCTION

In vitro experiments have demonstrated that secondary metabolite compounds of higher plants are able to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (*1*). Free radicals play an important role as causative agents in a variety of chronic diseases, such as cancer, aging (*2*), and Alzheimer's disease (*3*). Chemists have synthesized and extracted many effective agents to inhibit free radicals' oxidative damage from many different materials such as rubber, plastics, and foodstuffs. Due to the toxicity of artificial antioxidants, natural ones are more attractive to us, especially those from Chinese herbal medicine. Several kinds of natural components in plants possess antioxidant activities, such as flavonoids (*4*), anthocyanins (*5*), and phenolic compounds (*6*−*8*). Species of *Dioscorea* are some of the most famous medical herbs. Hot water extraction, steam distillation, and ethanolic solvents at ambient condition are the most useful methods traditionally employed to extract functional compounds from those medical herbs.

Fluid under pressure above its boiling point, but below its critical temperature, is called a hot pressurized solvent (sometimes called a subcritical solvent). Pressure applied to the extraction has to be higher than the saturated vapor pressure of that fluid to maintain it at liquid state. Under hot enough and high-pressure conditions, water can extract more nonpolar and lipophilic compounds than it can at room temperature. The temperature effect is more significant than pressure in changing the dielectric constant (polarity) of water (*9*), and the dielectric constant will decrease with respect to the increase in temperature. In recent years, hot pressurized water has been used to extract a few natural plant materials (*10*−*12*). This study investigates the free radical scavenging ability of compounds from Taiwan yams extracted by using semicontinuous hot pressurized solvents.

MATERIALS AND METHODS

Plant Materials. Three Taiwan varieties of *Dioscorea alata* tubers (Darsan, Tainung #2, and *Purpurea-Roxb*) were furnished by Ming-Jian Shiang Farmers' Association, Nantou Shian, Taiwan.

* Corresponding author (telephone +886 4 22852592; fax +886 4 22860231; e-mail cmchang@dragon.nchu.edu.tw).
† Department of Chemical Engineering.
§ Department of Chemistry.

10.1021/jf0351826 CCC: $27.50
Published on Web 03/06/2004

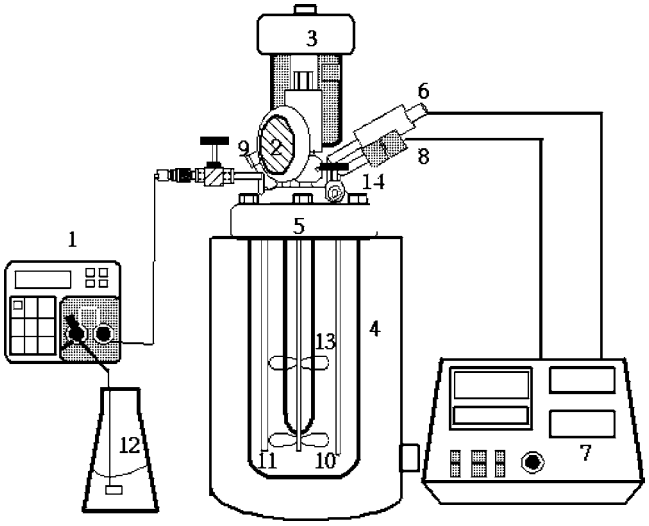

Figure 1. Schematic flow diagram of the experimental apparatus: 1, LC pump; 2, pressure gauge; 3, motor; 4, heater; 5, extractor; 6, pressure detector; 7, temperature controller; 8, thermocouple; 9, safety rupture disk; 10, internal string system; 11, inlet tube; 12, sample bottle; 13, cooling coil; 14, outlet valve.

Partition of Compounds Scavenging 1,1-Diphenyl-2-picrylhydrazyl (DPPH) Free Radical. All dried crude bodies were divided into meat and peel portions. A high-speed mixer ground them down; pieces under the standard size of no. 40 mesh (~420 μm) were collected and stored in a vacuum container before use. Twenty grams of the powders was extracted in a 170 mL Soxhlet extractor with ethanol (95%, v/v) under reflux for 3 h; each sample was extracted in duplicate by fresh ethanol at the boiling point, and then the extract solution was combined and concentrated under vacuum to remove the ethanol to obtain the aqueous dissolution of crude ethanol extracts. It is necessary to add >500 mL of water to completely dissolve the solute after the removal of ethanol. The aqueous concentrate of crude ethanol extract from the Soxhlet process was extracted in succession individually with hexane (H), chloroform (C), ethyl acetate (EA), and *n*-butanol (B) and further separated into five fractions using liquid−liquid (L−L) partitions. The final volume of each fraction was 300 mL based on triplicate experiments of 100 mL for each. Solvent polarity from the lowest to the highest is hexane < chloroform < ethyl acetate < *n*-butanol; also, it is true for the compounds extracted from each solvent. Each organic solvent was contacted with aqueous solution in the ratio of 1:1 (v/v). Five fractions and crude ethanol extract were ready to treat for the detection of scavenging activity on the DPPH free radical.

Hot Pressurized Solvent (HPS) Extraction. HPS extraction was performed in a 1 L stirred extractor. **Figure 1** depicts a schematic flow diagram of the experimental apparatus. The extraction system consisted of one JASCO model PU-1580 intelligent pump (JASCO, Tokyo, Japan) delivering solvent at a constant flow rate (10 mL/min) to a Parr 4520 autoclave linked with a Parr 4842 temperature controller (Parr, Moline, IL). To ensure that the solvent was in the liquid state at all of the temperatures tested, an SS-31RF2 regulating valve was placed at the outlet of the autoclave to maintain the system pressure at 300 psig (2.170 MPa) for hot pressurized water (HPW) extractions and from 80 to 280 psig (0.653−2.032 MPa) for hot pressurized ethanol (HPE) extractions. The autoclave was initially filled with a certain amount of yam peel and solvent and then mounted vertically, and solvent was pumped flowing from bottom to top.

For studying the effect of temperature, the extractor was preheated to the required temperature before solvent was pumped. When the system achieved the required pressure, the extraction time was reset to start. For doing solid loading experiments, the system temperature was selected as 120 °C, on the basis of the best result of several temperature−effect experiments. The solid loadings (solvent-to-feed ratio) were 13.9, 18.0, 25.7, and 36.0 kg/kg, and all extractions were carried out for 3 h. After the on-time sampling, the scavenging ratio (SR, %) of the extract was determined by eq 1 to demonstrate the percentage of DPPH free radical being scavenged.

$$\mathrm{SR}\ (\%) = \frac{(\mathrm{ABS_{blank}} - \mathrm{ABS_{reacted}})}{\mathrm{ABS_{blank}}} \times 100\% \qquad (1)$$

$\mathrm{ABS_{blank}}$ is the absorption value of DPPH in the background solvent. $\mathrm{ABS_{reacted}}$ is the absorption value of DPPH reacted with sample.

Test of DPPH Free Radical Scavenging. Detection of DPPH scavenging activity was carried out according to the method of Mensor et al. (*13*) with slight modification. The absorption value was measured and recorded by a U-3000 UV−vis spectrometer (Hitachi, Tokyo, Japan) at 517 nm, and the value had not decreased sharply after 30 min. The EC_{50} value was defined as the concentration of the sample sufficient to reduce to 50% the maximum absorption value estimated in the blank DPPH test. Besides, the SR value was another index of DPPH scavenging ability different from the EC_{50} value. It represents the percentage of DPPH free radical being scavenged and is calculated by eq 1. Using the SR value is more convenient to determine the antioxidant ability level, but it is not as precise as the EC_{50} value.

HPLC Quantification of Important Compounds. The equipment for HPLC analysis consists of a Waters 600E pump, a Waters 486 UV detector, a Waters 717 plus autosampler, and Millennium 2010 processing software. **Table 1** shows a gradient solvent program to analyze nine flavonoids, but an isocratic solvent program with 25% methanol and 75% deionized water (0.1% H_3PO_4 aqueous buffer solution) was used to analyze phenolic acids. A reverse phase Macherey-Nagel Nucleosil C-8 column (250 × 4.6 mm i.d.) was used, and the Waters 486 UV detector wavelength was set at 280 nm.

Experimental Design. The Taguchi method investigated in this study was taken from Chen et al. with a slight modification (*14*). Four parameters (temperature, pressure, ethanol content, and solvent-to-feed ratio) and a three even space level (−1, 0, and 1) L_9 orthogonal array was allocated for the evolution of a semicontinuous HPE extraction of DPPH scavenging compounds, expressed as the SR ratio. This experimental design was configured to find which of four controllable parameters is the major influencing parameter. **Table 2** shows the corresponding parameters and levels.

Table 1. Gradient Program of HPLC Analysis for Nine Flavonoids

time (min)	0.1% H_3PO_4 (%)	MeOH (%)
0	65	35
15	50	50
35	50	50
50	35	65
51	0	100
60	0	100

Table 2. Parameters and Levels of the L_9 Experimental Design

	parameters			
no. (*j*)	*T* (°C), A	*P* (psig)/(MPa), B	ethanol (%), C	S/F (kg/kg), D
1	80	80/(0.653)	65	20
2	80	180/(1.342)	80	30
3	80	280/(2.032)	95	40
4	100	80/(0.653)	80	40
5	100	180/(1.342)	95	20
6	100	280/(2.032)	65	30
7	120	80/(0.653)	95	30
8	120	180/(1.342)	65	40
9	120	280/(2.032)	80	20

RESULTS AND DISCUSSION

Partition of DPPH Free Radical Scavenging Compounds. **Table 3** lists the EC_{50} values of five partitions produced from four L−L extractions and crude ethanolic extract for peel and meat portions of three Taiwan yams. Because the crude ethanol extract (E) contains more complicated substances, results for a

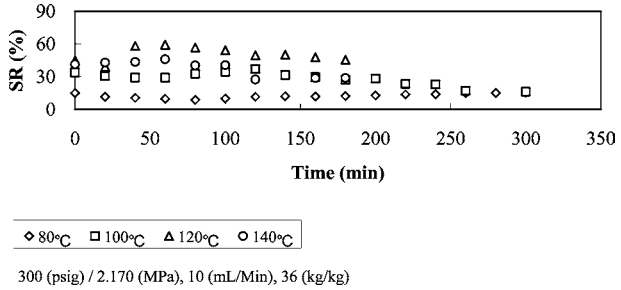

Figure 2. Temperature effect on DPPH scavenging ratio in HPW extraction of *Purpurea-Roxb* peel.

Table 3. EC_{50} (Micrograms per Milliliter) of Six Partitions Formed by L–L Extractions for the Extracts of Three Yams

sample		partitions[a]					
		E	H	C	EA	B	W
Tainung #2	meat	ND[b]	2360.1	549.4	320.5	573.8	ND
	peel	86.6	133.0	45.8	14.5	88.4	ND
Darsan	meat	ND	328.0	144.6	343.6	288.1	ND
	peel	305.9	932.7	130.2	38.8	678.3	ND
Purpurea-Roxb	meat	ND	604.5	359.8	112.8	1525.6	ND
	peel	130.4	236.2	136.6	23.8	67.2	ND
vitamin C				21.4			

[a] E, ethanolic crude; H, hexane; C, chloroform; EA, ethyl acetate; B, *n*-butanol; W, water. [b] ND, not detected.

few very effective compounds are presented in lower mole fraction than those of some organic solvent extracts (C, EA, and B). Data also indicated that the remaining aqueous partition (W) did not show any scavenging effect on DPPH free radical after four L−L extractions. All of the effective antioxidants were totally extracted by the four organic solvents from the aqueous solution, which revealed that the antioxidants were more easily soluble in organic solvents than in water. Furthermore, antioxidants are more easily dissolved in low to middle polarity solvents, as evidenced by the fact that the lowest EC_{50} value was found in the EA partition, which obtained from 14.5 to 38.8 μg mL^{-1}, and the Tainung #2 values were even lower than those for vitamin C (21.4 μg mL^{-1}). Another important finding is that the scavenging activities of peel portions were significantly better than meat portions in most cases. The peel of *Purpurea-Roxb* was chosen for use in the following semicontinuous experiments.

Hot Pressurized Solvent Extraction. Figure 2 depicts the temperature effect on DPPH free radical scavenging ratio (SR) of *Purpurea-Roxb* peel portion extracted using the semicontinuous HPW process. The point 0 min was set to start the semicontinuous process while temperature and pressure attained a desired condition. Before point 0, the system is similar to a batch extractor. Extraction conditions were suitable at 300 psig (2.170 MPa), 120 °C, and a 36.0 kg/kg solvent-to-feed (S/F) ratio. The DPPH free radical capture can reach 60%. Peels might be scorched when the temperature was approached 140 °C, which was revealed by a lower SR value and the black solid appearance after extraction. Extraction was also limited at the times <180 min because of the decreasing trend of the scavenging ratio.

Figure 3 indicates the effect of solid loadings on DPPH scavenging ratio in HPW extraction at 120 °C, 300 psig (2.170 MPa), and 10 mL/min. According to fixed vessel volume and constant flow rate, the final volume of each experiment is determined. In other words, the S/F ratios are solely dependent on the solid quantity. Four different S/F ratios (36.0, 25.7, 18.0, and 13.9) were used; the 25.7 (kg/kg) S/F ratio presents the most important results, with the highest DPPH scavenging ratio of up to 71.15%.

Ethanol was used as another solvent of semicontinuous HPS extractions. The extraction conditions were still at 120 °C, 300 psig (2.170 MPa), 10 mL/min, and 25.7 (kg/kg) S/F ratio. Our results showed that HPE has a better free radical scavenging ability than water under the same conditions, especially 80% ethanol, as shown in **Figure 4**. EA and water-saturated ethyl acetate (WS-EA) were also used as solvents, but the extract could not capture the free radical as compared to ethanol.

HPLC Identification of Four Unknown Antioxidants. Nine important flavonoids (naringene, quercetin, kaempherol, pinocembrin, isorhamnetin, caffeic acid phenethyl ester, galangin, chrysin, and acacetin) were screened and analyzed by HPLC. Another four typical compounds (phenolic acids) usually treated as antioxidants were also analyzed by HPLC. Comparison between the HPE extract chromatogram with standards implies that the nine flavonoids and four phenolic acids were not found in the extracts. Inspecting the HPLC chromatogram of HPE samples revealed that there are four peaks (U1−U4) having absorption strength positively related to the scavenging ratio. **Figure 5** shows the similar trend between the scavenging ratio and the recovery of four unknowns in HPE extracts. It can be concluded that all four unknown compounds were effective antioxidants with the ability to scavenge DPPH free radicals. The percentage content of four unknowns in four separation fractions of L−L extraction revealed that the most effective antioxidative ability was found in the EA fraction as compared with the other three fractions (H, C, and B). Summing the area

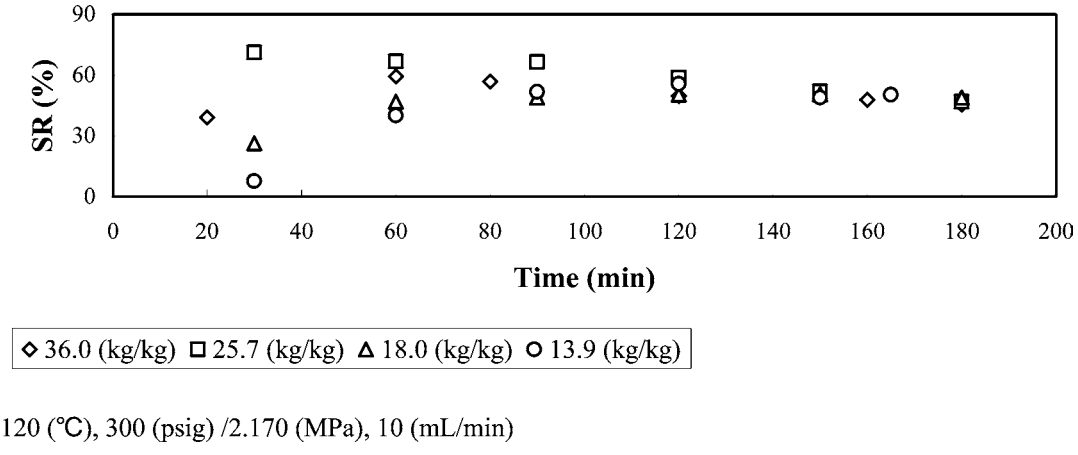

Figure 3. Loading effect on DPPH scavenging ratio in HPW extraction of *Purpurea-Roxb* peel.

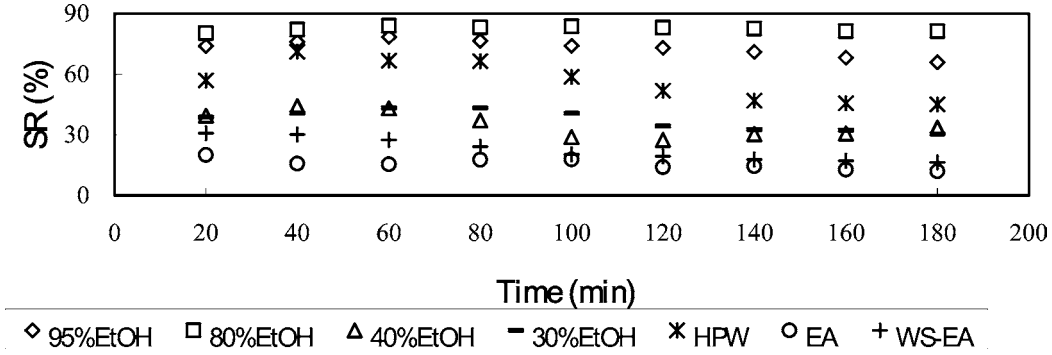

Figure 4. Comparison between hot pressurized solvents and HPW extraction of *Purpurea-Roxb* peel.

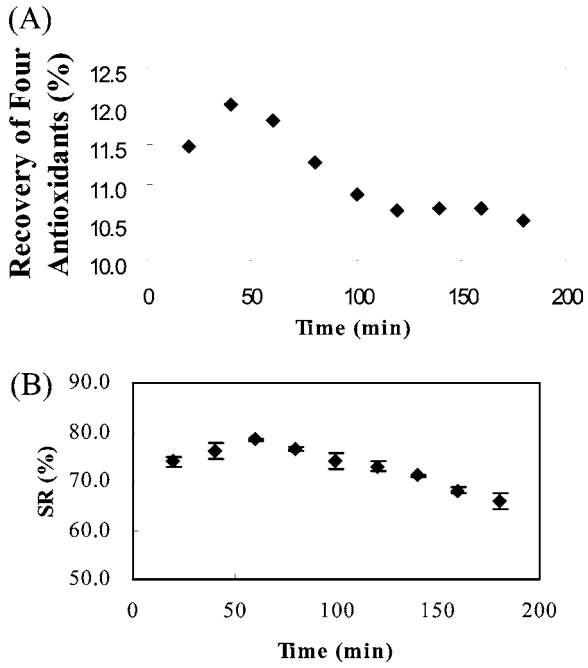

Figure 5. Recovery of four unknown antioxidants (**A**) corresponding with the scavenging ratio of DPPH (**B**); HPE20 to HPE180 indicate samples of the hot pressurized ethanol extraction collected at time 20–180 min. The area of four peaks in the HPLC chromatogram calculates the recovery.

Table 4. Content of Four Antioxidants Distributed in L–L Solvent Fractions and Hot Pressurized Solvent Extraction of *Purpurea-Roxb* Peel

	antioxidants (wt %)				
sample	U1	U2	U3	U4	yield (g/100 g)
hexane	ND[a]	ND	ND	ND	0.45
chloroform	ND	2.2	ND	76.3	0.43
EA	**12.3**	**4.2**	**74.0**	**9.5**	0.23
n-butanol	59.2	40.8	ND	ND	0.40
HPE	11.8	12.9	9.2	1.7	2.94
HPW	28.8	3.8	ND	ND	21.43

[a] ND, not detected.

of the four peaks shown in the HPLC spectra of each fraction disclosed that these four unknowns were the major compounds existing in the EA fraction, especially U3. U4 existed in only the C fraction, and U1 and U2 existed in only the B fraction, as shown in **Table 4**. It is difficult to obtain the recovery of these four unknowns for each fraction because the mass balance of five fractions is involved. However, the recovery of four unknowns could be calculated by weight for the extracts of HPE and HPW. Although having a lesser percentage than that in the

Table 5. ANOVA Analysis of Four Parameters for Hot Pressurized Ethanol Extraction

source	sum of squares (SS)	degrees of freedom (D)	variance (V)	*F* value (F)	$F_{0.05}$ (2,2)	$F_{0.01}$ (2,2)
total	4171.32	8				
(A) temperature	923.85	2	461.92	4.33	19.00	99.00
(B) pressure	769.67	2	384.83	3.60		
(C) EtOH ratio	2264.28	2	1132.14	10.60		
(D) S/F ratio	213.53	2	106.76			

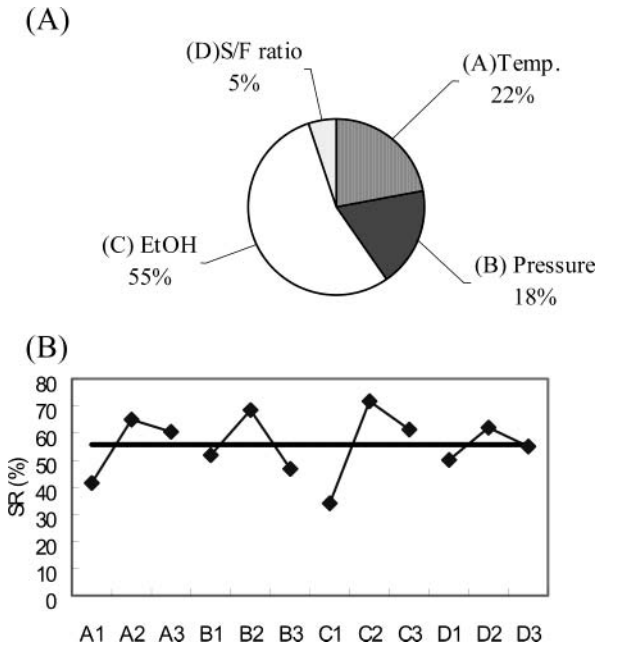

Figure 6. ANOVA percentage of the scavenging ratio (**A**) and the average value on each level (**B**).

EA fraction, it indeed showed that only HPE could extract the four unknowns in the semicontinuous process. This study has established and demonstrated a semicontinuous HPS process to extract antioxidant-capable compounds from *Dioscorea* yams in the engineering point of view. Biotests and identification of these four unknowns will be a follow-up subject.

Significant Parameters Obtained from Experimental Design Study. Table 5 lists the data of the analysis of variance (ANOVA) table of this experiment. **Figure 6** shows the percentage contribution of each parameter. According to the ANOVA table of the SR value, the *F* value ($F_c = 10.6$) and the contribution ($\rho_c = 55\%$) of ethanol content are larger than other parameters, and temperature and pressure ($F_A = 4.33$, $\rho_A = 22\%$; $F_B = 3.6$, $\rho_B = 18\%$) were not significant in this situation, but we still could not neglect the contribution from

these two parameters because the difference among parameters C, A, and B was not large enough. It might be that ethanol was the most significant parameter and temperature was the second. The free radical scavenging ratio was determined well in the extract of 80% ethanol, 100 °C, 20.0 (kg/kg) solid ratio, and 180 psig (1.342 MPa), which were obtained from this experimental design.

CONCLUSIONS

Liquid—Liquid Extractions. L−L extractions and test of DPPH free radical scavenging were used in this study to find the right solvents to obtain antioxidants extracted from three Taiwan yams. The ethyl acetate fraction contains the most effective antioxidant scavenging the DPPH free radical (i.e., the lowest EC_{50} value).

Both peel and meat portions of three Taiwan yams possess a few middle−high polar antioxidants, and the peel portion contains more active antioxidant than the meat portion.

Hot Pressurized Solvent Extractions. Suitable conditions for the semicontinuous HPW extractions of DPPH free radical scavenging antioxidants from *Purpurea-Roxb* yam peel were found at 120 °C, 300 psig (2.170 MPa), and 25.7 (kg/kg) S/F ratio in a 1 L extractor.

High-pressurized ethanol extraction is much better than HPW extraction in extracting four unknown antioxidants.

Four unknown antioxidants extracted from the peel portion of *Purpurea-Roxb* yam (*Dioscorea*) were found in a strong correspondence of DPPH free radical scavenging ability. However, nine important flavonoids and four common phenolic acids were not found in the extract.

Results of the L_9 experimental design disclosed that a suitable extraction condition was found using 80% ethanol as solvent and an extraction temperature of 100 °C. The content of ethanol and temperature might be significant operational parameters in scavenging the DPPH free radical. Pressure and solvent-to-feed ratio were not major factors.

LITERATURE CITED

(1) Larson, R. A. The Antioxidants of Higher Plants. *Phytochemistry* **1998**, *27*, 969−978.

(2) Ames, B. N.; Shigena, M. K.; Hegen, T. M. Oxidants, Antioxidants, and the Degenerative Diseases of Aging. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 7915−7922.

(3) Smith, M. A.; Perry, G.; Richey, P. L.; Sayre, L. M.; Anderson, V. E.; Beal, M. F.; Kowal, N. Oxidative Damage in Alzheimer's. *Nature* **1996**, *382*, 120−121.

(4) Cao, C.; Sofic, E.; Prior, R. L. Antioxidant and Prooxidant Behavior of Flavonoids: Structure−Activity Relationships. *Free Radical Biol. Med.* **1997**, *22*, 749−760.

(5) Espin, J. C.; Soler-Rivas C.; Wichers, H. J.; Garcia-Viguera, C. Anthocyanin-Based Natural Colorants: A New Source of Antiradical Activity for Foodstuff. *J. Agric. Food Chem.* **2000**, *48*, 1588−1592.

(6) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Antioxidant Properties of Phenolic Compounds. *Trends Plant Sci.* **1997**, *2*, 152−159.

(7) Kähkönen, M. P.; Hopia, A. I.; Vuorela, H. J.; Rauha, J. P.; Pihlaja, K.; Kujala, T. S.; Heinonen, M. Antioxidant Activity of Plant Extracts Containing Phenolic Compounds. *J. Agric. Food Chem.* **1999**, *47*, 3954−3962.

(8) Kähkönen, M. P.; Hopia, A. I.; Heinonen, M. Berry Phenolics and Their Antioxidant Activity. *J. Agric. Food Chem.* **2001**, *49*, 4076−4082.

(9) Miller, D. J.; Hawthorne, S. B. Method for Determining the Solubilities of Hydrophobic Organics in Subcritical Water. *Anal. Chem.* **1998**, *70*, 1618−1621.

(10) Ibañez, E.; Kubátová, A.; Señoráns, J. F.; Cavero, S.; Reglero, G.; Hawthorne, S. B. Subcritical Water Extraction of Antioxidant Compounds from Rosemary Plants. *J. Agric. Food Chem.* **2003**, *51*, 375−382.

(11) Clifford, A. A.; Basile, A.; Al-Saidi, S. H. R. A Comparison of the Extraction of Clove Buds with Supercritical Carbon Dioxide and Superheated Water. *Fresenius' J. Anal. Chem.* **1999**, *364*, 635−637.

(12) Chang, L. H.; Cheng, Y. C.; Chang, C. J. Extracting and Purifying Isoflavones from Defatted Soybean Flakes Using Superheated Water at Elevated Pressures. *Food Chem.* **2004**, *84*, 279−285.

(13) Mensor, L. L.; Menezes, F. S.; Leitão, G. G.; Reis, A. S.; Santos, T. C.; Coube, C. S.; Leitão, S. G. Screening of Brazilian Plant Extracts for Antioxidant Activity by Using of DPPH Free Radical Method. *Phytother. Res.* **2001**, *15*, 127−130.

(14) Chen, R. S.; Chen, Y. M.; Chiu, K. C. Effects of Experimental Parameters on the Yield of C_{60} Powder Studied by Taguchi Method. *Chin. J. Mater. Res.* **1999**, *31*, 127−134.

Received for review October 13, 2003. Revised manuscript received January 30, 2004. Accepted February 3, 2004. We thank the National Science Council of the Republic of China for financial support of this research under Contract NSC92-2214-E005-003.

JF0351826